Role of Notch Signaling in Human T Cell Activation using Artificial Antigen Presenting Cells

by

Milica Tanic

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Immunology
University of Toronto

© Copyright by Milica Tanic 2016
Role of Notch Signaling in Human T Cell Activation using Artificial Antigen Presenting Cells

Milica Tanic

Master of Science

Department of Immunology
University of Toronto

2016

Abstract

Adoptive cell transfer of ex vivo-expanded tumor infiltrating lymphocytes (TILs) is an emerging approach for treating cancers. One limitation in this approach is the use of natural antigen presenting cells (APCs) for TIL expansion. Unlike natural APCs, “artificial” APCs (aAPCs) are easily accessible and manipulated cell lines shown to support priming and activation of tumor-associated antigen-specific CD8⁺ cytotoxic T lymphocytes. We hypothesize that providing Notch signaling during priming and activation using aAPCs will generate CTLs better suited for immunotherapy and long-term tumor regression. K562 erythroleukemia-derived aAPCs were genetically modified to express the Notch ligand, Delta-like-4. Here, we report that provision of Notch signaling during suboptimal priming of human naïve umbilical cord-derived CD8⁺ T cells supports higher frequencies of activated T cells indicated by CD25⁺ or CD71⁺ cells. Having optimized conditions for revealing the effects of DLL4 in early activation, future experiments will determine their impact on anti-tumor functionality.
Acknowledgments

I would like to thank my supervisor, Dr. Juan Carlos Zúñiga-Pflücker for providing me with the opportunity to conduct biological research in an enriching and stimulating environment. His support and guidance were instrumental to my success as a graduate student and young scientist. Over the past two years, I have grown tremendously both academically and personally and have gained invaluable skills that will last a lifetime. I hope to be able to continue as a member of the scientific community and to contribute to cancer immunotherapy research for the remainder of my career.

I would also like to thank the ambitious and hard-working members of the Zúñiga-Pflücker lab, both past and present, who helped me to master key techniques, skills and approaches to experimental design. I would especially like to thank Drs. Patricia Benveniste and Mahmood Mohtashami for their patience and support, without whom my journey would have been difficult. In addition I would like to thank the Department of Immunology and the School of Graduate studies for funding my work, partly through the Ontario Graduate Scholarship.

I would like to thank my committee members Drs. Naoto Hirano and Pamela Ohashi for their insight and suggestions both during and outside our meetings, which were important for the evolution of my project.

Finally, I would like to thank my family and friends for their support over the course of my degree.

This thesis contains original work and experiments designed and performed by me with input from Drs. Juan Carlos Zúñiga-Pflücker, Patricia Benveniste, Mahmood Mohtashami, Munehide Nakatsugawa, Naoto Hirano and Pamela Ohashi. Drs Naoto Hirano, Pamela Ohashi and Carl June provided me with critical reagents, namely the ABC and K80 artificial antigen presenting cell lines and cell culture materials. I would also like to thank Noriko Nakatsugawa for having created the K80 DLL4 artificial antigen presenting cell line.
# Table of Contents

Acknowledgments .................................................................................................................... iii

Table of Contents .......................................................................................................................... iv

List of Tables ............................................................................................................................... vi

List of Figures ............................................................................................................................. vii

Chapter 1 Introduction ................................................................................................................. 1

The Notch pathway ....................................................................................................................... 1

Notch in T cell development ....................................................................................................... 4

Notch in the peripheral immune system ...................................................................................... 5

T cell biology ............................................................................................................................... 8

Immunotherapy ............................................................................................................................ 10

Artificial antigen presenting cells ............................................................................................... 13

Hypothesis and Thesis Focus ..................................................................................................... 14

Chapter 2 Materials and Methods ............................................................................................. 16

Umbilical cord blood processing ............................................................................................... 16

CD34\(^+\) stem cell preparation ................................................................................................. 16

CD8\(^+\) T cell harvesting ........................................................................................................... 17

aAPCs ........................................................................................................................................ 17

aAPC transduction ...................................................................................................................... 19

T cell development from CD34\(^+\) CD38\(^-\) stem cells ............................................................... 19

CD8\(^+\) T cell activation ............................................................................................................ 20

Flow cytometry .......................................................................................................................... 20

Quantitative RT-PCR ................................................................................................................ 22

Statistical analyses .................................................................................................................... 22

Chapter 3 Results ....................................................................................................................... 24
aAPCs can be efficiently transduced to express biologically functional human DLL4 ....24

DLL4 expression by aAPCs leads to increased frequencies of CD25+ and CD71+ CD8+ T cells during early priming and activation..............................................................28

Variable effect of DLL4 on CD8+ T cell activation in different UCB samples ..............33

Stimulation of CD27 leads to further increased frequencies of CD25+ and CD71+ cells in presence of DLL4 on aAPCs ........................................................................................................35

Effect of DLL4 in T cell transcriptional changes during priming and activation..........38

Chapter 4 Discussion ............................................................................................................40

List of Abbreviations ..............................................................................................................50

References............................................................................................................................52
List of Tables

Table 1 .................................................................................................25

Table 2 .................................................................................................28

Table 3 .................................................................................................30
List of Figures

Figure 1 ........................................................................................................9

Figure 2 .......................................................................................................10

Figure 3 .....................................................................................................18

Figure 4 .....................................................................................................34

Figure 5 .....................................................................................................37

Figure 6 .....................................................................................................39

Figure 7 .....................................................................................................41

Figure 8 .....................................................................................................44

Figure 9 .....................................................................................................46

Figure 10 ...................................................................................................56
Chapter 1

Introduction

The Notch pathway

The Notch signaling pathway is an evolutionarily conserved pathway involved in directing many different developmental programs, including angiogenesis, hematopoiesis and neurogenesis\(^1,2\). The Notch family of receptors consists of four members (Notch 1-4), which are heterodimeric receptors made up of an extracellular ligand-binding domain non-covalently associated with a transmembrane polypeptide domain\(^3\). Notch receptors are glycosylated type 1 transmembrane glycoproteins consisting of several distinct structural domains, such as several epidermal growth factor (EGF)-like repeats, a negative regulatory region (NRR) and a heterodimerization domain (HD) all in the extracellular domain\(^4,5\). Specific functions are attributed to domains including regulation of interactions with ligands by EGF-like repeats, and prevention of ligand-independent activation by the NRR\(^6\). The intracellular domain of Notch (NICD) contains motifs such as nuclear localization signals (NLS) and several ankyrin repeats comprising the Ankyrin domain (ANK), which are important for binding to transcriptional machinery, and transcriptional function of the NICD requires the transactivation domain (TAD)\(^6,7\). Notch receptors bind members of the Delta family (Delta-like 1, 3 and 4) or Serrate family (Jagged 1 and 2), which are membrane-bound proteins found on a variety of cell types (Figure 1)\(^3\).
Figure 1: Notch ligands and receptors. There are four Notch receptors (Notch 1-4), each consisting of an extracellular ligand-binding domain non-covalently associated with a transmembrane polypeptide domain. The extracellular portion contains Epidermal Growth Factor-like (EGF-like) repeats and the intracellular portion contains a Heterodimerization Domain (HD), an RBP-J-associated Molecule Domain (RAM), an Ankyrin Domain (ANK), a Transactivation Domain (TAD), a Proline-, Glutamate-, Serine- and Threonine-rich Domain (PEST) domain, and a Nuclear Localization Sequence (NLS). Notch receptors bind members of the Delta family (Delta-like 1, 3 and 4) or Serrate family (Jagged 1 and 2), which are membrane-bound proteins containing EGF-like repeats and conserved cysteine-rich (CR) and Delta/Serrate/Lag (DSL) domains.

Adapted from: Osborne and Minter 2007, Notch signaling during peripheral T-cell activation and differentiation. doi:10.1038/nri1998

Notch receptors are synthesized as single proteins, but in the cytoplasm undergo S1 cleavage by a furin-like convertase that yields the final heterodimeric protein. While each Notch receptor preferentially binds a certain ligand, for example Notch 1 and DLL4, glycosylation of EGF-like repeats by Fringe glycosyl transferases (Lunatic, Manic and Radical Fringe) can enhance or reduce binding of the Notch receptor to a given ligand. Upon ligation, Notch receptors undergo proteolytic cleavage first by ADAM family metalloproteases (S2 cleavage) followed by a γ-secretase complex containing presenilin, nicastrin, APH-1 and PEN-2 in the
inner surface of the plasma membrane (S3 cleavage) resulting in release of the NICD (Figure 2)\textsuperscript{3, 6, 10, 11}.

Figure 2: The Notch Pathway. A Notch ligand associates with the extracellular domain of a Notch receptor, exposing S2 for cleavage by ADAM family proteases\textsuperscript{5}. Cleavage at the intracellular membrane occurs at S3 by a $\gamma$-secretase complex, releasing the Notch intracellular domain (NICD) into the cytosol\textsuperscript{6}. NICD translocates into the nucleus where it associates with CSL and transcriptional co-activators to induce transcription of Notch target genes\textsuperscript{6}. \textbf{Adapted from:} Radtke et al 2010. Notch Signaling in the Immune System doi: 10.1016/j.immuni.2010.01.004..

In order for Notch signaling to be induced in a receptor-bearing cell it is necessary to induce Notch ligand endocytosis in the ligand-bearing signal-sending cell, for which there are two proposed models. The first, The Recycling Model, features endocytosis of initially inactive Notch ligands on the surface of ligand presenting cells followed by intracellular modification.
and return to the cell surface for receptor engagement that renders the ligands active and able to induce Notch receptor activation\textsuperscript{6,12}. The Mechatransduction Model suggests that upon ligand-receptor engagement, endocytosis of the ligand causes pulling of the receptor that exposes S2 to ADAM proteases and allows generation of the NICD\textsuperscript{13}. The NICD translocates to the nucleus where it functions to activate transcription of target genes by associating with a helix-loop-helix transcription factor, known as RBPJκ (mouse), CBF-1 (human) or Suppressor of Hairless (\textit{Drosophila}), Lag1 (\textit{C. elegans}), and other co-activating molecules such as Mastermind-like 1 (MAML1) and p300 to form a transactivating complex inducing gene transcription\textsuperscript{2,6,11}.

Notch in T cell development

The Notch signaling pathway is absolutely required for the development of T cells, including commitment and specification to the T lineage\textsuperscript{4,14}. Upon emigration from the bone marrow, thymic seeding progenitor cells (TSPs) enter the thymus at the corticomedullary junction and subsequently undergo differentiation into mature T cells as they pass through various thymic compartments\textsuperscript{15}. TSPs entering the thymus are devoid of expression of CD4 and CD8, and are thus termed to be in the double negative (DN) stage\textsuperscript{6}. The DN stage can be further subdivided into four stages based on the expression of CD44 and CD25 progressing through the following order: DN1 (CD44\textsuperscript{−} CD25\textsuperscript{−}), DN2 (CD44\textsuperscript{+} CD25\textsuperscript{+}), DN3 (CD44\textsuperscript{−} CD25\textsuperscript{+}) and DN4 (CD44\textsuperscript{−} CD24\textsuperscript{+})\textsuperscript{6}. While the cells become committed to the T cell lineage by the DN3 stage, they must further pass through a series of positive and negative selection steps that ultimately culminate in the
development of single positive (SP) CD4$^+$ or CD8$^+$ T cells, which then emigrate to the periphery$^6$. Continuous Notch signaling is required until the DN3 stage for proper T cell development$^6$. In the thymus, it has been shown that though there are several Notch ligands, it is DLL4 that is absolutely required for T cell development$^{16}$. In the absence of DLL4 expression by thymic epithelial cells, B cells develop in the thymus$^{16}$. The highest levels of DLL4 expression in the thymus are found in the cortical thymic epithelial cells in the outer periphery of the thymus where DN cells are passing through early developmental stages$^6$. Jagged 1 and 2 can also be found in the adult thymus$^6$. DLL1 is also able to support T cell development in vitro although deletion of DLL1 in mice does not impair T cell development and DLL4 has been shown to be the more potent of the two in inducing Notch1 signaling$^{16,17}$.

Notch in the peripheral immune system

Besides the embryonic and adult thymus, Notch ligands are expressed on a variety of mature peripheral blood cell types and several groups have shown that the Notch pathway is important during mature T cell activation and differentiation$^3$. This is underscored by observations that antigen presenting cells (APCs) increase expression of Notch ligands upon toll-like receptor (TLR) activation$^{18}$. For example, DCs express Notch ligands that are involved in directing various T helper lineages$^{19}$. Unsurprisingly Notch receptors, mainly Notch 1 and Notch 2, are expressed on CD4$^+$ and CD8$^+$ T cells and expression varies according to antigen experience and activation status. Human naïve CD8$^+$ T cells express Notch 2 while established effector cells express Notch 1 and Notch 2$^{18}$. Others have reported that both Notch 1 and 2
are expressed at low levels on naïve CD4⁺ and CD8⁺ T cells and are upregulated upon activation. Upon T-cell receptor (TCR) ligation itself, Notch signaling is also induced on T cells and this is hypothesized to occur via hypoxia-inducible factor-1α mediated stabilization of the NICD. It has also been implicated in protection from TCR-induced cell death via inactivation of Nur77, which is pro-apoptotic. The role of the Notch pathway has been studied mostly in CD4⁺ T helper cell differentiation, and to a lesser extent in CD8⁺ T cells. It has been shown that Notch ligands on APCs during activation can instruct the differentiation of CD4⁺ and CD8⁺ T cells. Different Notch ligands can have opposing influences on activated T cell differentiation, such as DLL4 which promotes Th1 responses while Jagged ligands promote Th2 responses. Interestingly, in RBPJ-deficient cells Th2 differentiation was impaired while Th1 differentiation was intact, suggesting a non-canonical Notch pathway may be involved in Th1 differentiation. It is important to note that there is variability in the findings from study to study, which may be due to different APCs, activation conditions and other experimental factors.

Progress has been made in determining a function of Notch receptors in CD8⁺ T cell differentiation and function as well. Production of IFN-γ by mouse CD8⁺ T cells is inhibited in the presence of a γ-secretase inhibitor (GSI). Similarly in naïve human CD8⁺ T cells, a GSI or soluble DLL4-Fc leads to impaired production of IFN-γ, CD107a expression and cytolytic capabilities. Impaired Notch signaling in established effector cells prevents IFN-γ production but not cytolytic function as measured by CD107a mobilization. Furthermore, the expression of programmed cell death protein-1 (PD-1) in activated CD8⁺ T cells is also directly transcriptionally regulated by Notch signaling during activation indicating that there is a role
of Notch in later stages of CD8+ T cell function\textsuperscript{24}. Following activation, CD8+ T cells differentiate into short-lived effector cells (SLECs), which die during the contraction phase of the immune response, or memory precursor effector cells (MPECs), which will maintain immunological memory to the antigen\textsuperscript{22}. Deficiency of Notch 1 and 2 results in impaired differentiation of SLECs in mice during \textit{Listeria} infection hypothesized to be affecting the early effector to SLECs transition, a process requiring appropriate CD25 expression\textsuperscript{22}. Notably, MPEC differentiation was unimpaired in these mice suggesting that Notch is preferentially required for effector rather than memory cell differentiation\textsuperscript{22}. Blocking of Notch signaling \textit{in vivo} in CD4+ and CD8+ T cells prevents graft versus host disease indicating that the Notch pathway can have a very significant impact on systemic immune reactions\textsuperscript{25}. Furthermore, some of the effects of Notch signaling in CD8+ and CD4+ T cells have been shown to promote anti-tumor activity\textsuperscript{26}. A tumor mouse model showed that Notch 2, but not Notch 1, was required for antitumor activity and cytotoxic T-lymphocyte (CTL) differentiation in CD8+ T cells\textsuperscript{27}. In addition, antibody-mediated stimulation of Notch2 during activation of CD8+ T cells resulted in enhanced anti-tumor activity and survival compared to control suggesting Notch can participate in an additive or synergistic process during activation\textsuperscript{27}. When these cells were activated in the presence of Jagged 2, this effect was not recapitulated, indicating the ligand-specificity of the response\textsuperscript{27}. Suppression of DLL1 on activated DCs via shRNA caused impaired generation of CTLs and loss of Notch2 impaired production of Granzyme B and generation of CTLs\textsuperscript{28}. Finally, a recent study in mouse CD4+ T cells showed that the threshold for activation was reduced in the presence of DLL4 on APCs and that anti-tumor activity was significantly reduced when the T cells were activated by DLL4\textsuperscript{−/−} DCs\textsuperscript{18}. Together, these recent publications
support the notion that Notch signaling in T cells can modulate their effector function
differentiation and/or influence their activation.

T cell biology

Following exit from the thymus, in response to antigen presentation, naïve CD4+ and CD8+ T
cells become activated and differentiate into effector and memory cell types and contribute
to clearance of an infection and sustained memory for future reinfection. T cell activation
requires 3 signals: the first is TCR/CD3 ligation by a human leukocyte antigen (HLA) molecule
presenting an antigen, the second is co-stimulatory molecule-induced signaling and the third
comprises cytokines and is hypothesized to include Notch signaling2. Naïve CD8+ T cells
differentiate into effector T cells upon TCR ligation and co-stimulation and produce effector
cytokines including IFN-γ, Perforin and Granzyme B, as well as FAS ligand (CD95L), which
induces apoptosis via engagement of FAS on a target cell29. Besides serving as a necessary
signal to allow full priming and activation of a T cell, co-stimulatory molecules can affect the
survival, expansion and phenotype of activated T cells30. One such molecule is CD27, a
member of the Tumor Necrosis Factor (TNF) superfamily, which is expressed on naïve T cells
and following an initial increase in expression, decreases as activated T cells divide and
differentiate30. CD27 binds its ligand, CD70, which is expressed on activated dendritic cells
(DCs), T and B cells and can be regulated by various cytokines30. In mouse, CD27 promotes
activated T cell survival through induction of autocrine IL-2 production31.
Upon encountering antigen presented on class I HLA molecules in secondary lymphoid organs, naïve CD8+ T cells become activated and undergo clonal expansion. This division is supported by metabolic changes such as uptake of glucose, amino acids and iron. The phenotypes of the resultant T cells include: short-lived effector cells (SLEC), comprising the majority of the population, and memory precursor effector cells (MPEC) which will go on to establish the memory population. Differentiation towards the SLEC versus MPEC fate can be directed by different pre and post activation factors and both phenotypes can arise from a single starting CD8+ T cell. For example, interleukin-2 (IL-2) signaling preferentially promotes SLEC differentiation. Following activation, CD8+ T cells migrate into peripheral sites of infection to perform effector functions. CD8+ T cells also up-regulate negative regulatory molecules upon activation to limit the intensity and duration of effector function. A well-studied example is cytotoxic T-lymphocytes associated protein 4 (CTLA-4), which peaks in expression by day 2-3 post activation, and competitively prevents further T cell activation supported by CD28 co-stimulation by binding to CD28 ligands. In addition, TCR and IL-2 signaling induce PD-1 expression which binds its ligands PD-L1 and PD-L2 expressed on a variety of cells such as other T cells, myeloid cells and DCs. Blockade of both the PD-1 and CTLA-4 pathways has been investigated to determine whether effector cell function can be enhanced to promote anti-tumor immune function. Once infection is cleared, the cells enter a contraction phase characterized by massive death of effector cells while memory cells survive to provide memory for future infection.
Immunotherapy

At this time, the importance of the immune system in the development and progression of various malignancies has been acknowledged and is supported by various basic and clinical research findings. For example, improved clinical outcome correlates with tumor infiltrating lymphocytes (TILs) in malignancies such as metastatic melanoma, ovarian and breast cancer\textsuperscript{34}. There are a variety of immunotherapeutic approaches under current investigation for the treatments of cancers, some of which include adoptive cell transfer (ACT) of TILs or lymphocytes modified to express chimeric antigen receptors, immune checkpoint inhibitors (such as blocking CTLA4 and PD-1), and cancer vaccines. The various strategies have been met with differing degrees of success and this study specifically aims to improve ACT of TILs. Early research findings showing that TILs have anti-tumor activity in mouse and human studies spurred further investigation into harnessing the immune system to fight malignancies\textsuperscript{34}. For example, in animal models it has been shown that rejection of solid tumors is primarily mediated by CD8\textsuperscript{+} CTLs. Several clinical trials in metastatic melanoma have shown that administration of TILs and high dose IL-2 can induce objective responses in patients\textsuperscript{34}. Additional factors such as lympho-depletion conditioning and higher numbers of infused TILs also facilitated better outcome\textsuperscript{34}. In order to allow for expansion of TILs to population sizes necessary for therapeutic administration, original and some current methodology includes a rapid expansion protocol (REP) of tumor-reactive TILs\textsuperscript{34}. Briefly, REP features extraction of a tumor and plating of small tumor fragments in tissue culture plates in the presence of high dose IL-2 and human serum\textsuperscript{35}. Following 1-2 weeks, TILs grow out of these cultures and are
then assayed for anti-tumor activity using an IFN-γ-release assay\textsuperscript{35}. TIL cultures which exhibit anti-tumor function are expanded to the large population sizes needed for infusion into patients using anti-CD3 antibody, IL-2 and irradiated allogeneic feeder cells derived from healthy donors\textsuperscript{35} (Figure 3).

Figure 3: Current standard immunotherapy approach. Tumor fragments are excised from patients, then plated in small fragments and grown in high-dose IL-2 to expand tumor infiltrating lymphocytes (TILs)\textsuperscript{35, 36}. TILs are then selected for anti-tumor function and expanded using rapid expansion protocol (REP) to yield high numbers of TILs for infusion into patients\textsuperscript{35, 36}.

Adapted from: Rosenberg and Restifo 2015. Adoptive cell transfer as personalized immunotherapy for human cancer. doi: 10.1126/science.aaa4967.
While this protocol expands cell populations to therapeutic levels, it ultimately results in more differentiated T cells that display effector rather than memory phenotypes indicated by loss of CD62L and CCR7, as well as CD28 (Figure 3) \textsuperscript{37,38,39}. Initially, it was thought that the best cellular product would be one in which the T cells would show a more differentiated phenotype with the highest cytotoxic potential as measured by anti-tumor activity, however, animal studies have suggested that it may be best to infuse less differentiated TILs\textsuperscript{38,40}. Established effector cells are responsible for initial clearance of the tumor bulk, however, for sustained responses including remission, it is necessary to provide cells that are able to proliferate and establish immunological memory\textsuperscript{38,41}. Cells that are able to do this are memory cells and exhibit a less differentiated phenotype, contain longer telomeres and express markers such as CD62L and CCR7, CD27, IL-7 Receptor α chain (IL-7Rα), some of which allow them to enter memory compartments\textsuperscript{41,42}. When compared to differentiated effector cells, they are less able to produce IFN-γ and effector molecules, however, they can proliferate more and thus can support a long-term anti-tumor immune system\textsuperscript{38}. After the necessary several rounds of expansion, resultant T cells are often not able to provide sustained immune protection due to their limited ability to proliferate further\textsuperscript{43}. However, this effect can be avoided by shorter culture times and results in favourable patient responses\textsuperscript{34,38,42}. To further complicate the matter, the protocol is met with practical limitations pertaining to feeder cells. These are tedious to harvest and required in large numbers from many donors, imparting batch-to-batch variability and inconsistencies in the ability to expand TILs\textsuperscript{44}. This effectively prevents the systematic application of immunotherapy to patients in an efficient manner. Additionally, this technology is costly and
thus efforts have been made to create standardized and economical approaches to therapy generation.44

Artificial antigen presenting cells

In order to overcome difficulties and pitfalls pertaining to cells and cytokines used to activate and expand T cells, the use of K562-based artificial antigen presenting cells (aAPCs) has been explored by several groups. Derived from a patient with chronic myelogenous leukemia in blastic crisis, the K562 line is suitable for use in expansion of T cells.38 These cells express adhesion molecules such as intercellular adhesion molecule 1 (ICAM1 also known as CD54) and lymphocyte function-associated antigen 3 (LFA-3 also known as CD58) however, they do not express HLA proteins, and will thus not induce allogeneic responses.37, 43, 45 They have also been reported to express CD80 and B7-H3 and secrete IL-6, IL-8, monocyte chemotactic protein-1, macrophage inflammatory protein-α and transforming growth factor beta.137 Furthermore, they can be genetically modified to express key human immunological markers such as class I and II Human Leukocyte Antigen (HLA-I and HLA-II) proteins and can stably express a variety of classical T cell co-stimulatory molecules, such as CD137 ligand (also known as 4-1BB ligand (4-1BBL))45. 4-1BBL is considered an important co-stimulatory molecule for activation of CD8+ T cells and when expressed on aAPCs supports expansion of T cells specific for antigens of varying immunogenicity, long-term culture and maintenance of TCR repertoire diversity.43, 46 Hence, the aAPC system is plastic and can be optimized to yield desired immune responses. When expressing a class I HLA protein and pulsed with a peptide,
aAPCs can support expansion of healthy donor and patient-derived CD8+ T cells specific for tumor associated antigens such as melanoma antigen recognized by T cells (Mart-1), as well as polyclonal expansion of ovarian cancer TILs37, 44. The resultant cells are long-lived and exhibit cytotoxic activity against target cells and supplementing the cultures with cytokines such as IL-2 and IL-15 promotes increased IFN-γ production when activating naïve T cells37. Importantly, an aAPC-based system is able to expand TILs as well as REP in a clinical grade system in a manner independent of exogenously added cytokines thus this is a very feasible alternative for the clinic 45. Given that they are a cell line and easily maintained, K562 cells have been suggested as a suitable line to serve as a backbone of an “off-the-shelf” product that can be used to support expansion of T cells and would allow for standardization of therapeutic protocol implemented for each patient, absolving variability due to feeder cells 37, 47.

Hypothesis and Thesis Focus

Based on evidence indicating that: Notch signaling reduces the threshold for activation and is required for anti-tumor function of T cells in some models, in this study I hypothesized that the addition of Notch signaling during aAPC-based CD8+ T cell activation would facilitate a superior immunotherapy product and more cells would be activated initially, reducing rounds of activation required for expansion and preserving lymphocyte “youth”. In order to test this hypothesis, I made use of CD8+ T cells derived from human umbilical cord blood (UCB), upwards of 95% of which exhibit a naïve phenotype (CD45RA+ CCR7+ CD27+)48. Adult blood
(AB)-derived cells exhibit a more differentiated phenotype\textsuperscript{48} and so UCB-derived cells can provide an understanding of the effect of Notch signaling during truly naïve T cell priming and activation. Furthermore, as Mart-1-specific T cells are also found in UCB, it would be possible to study the effects of Notch signaling in both polyclonal and antigen-specific activation\textsuperscript{48}. Like AB-derived cells, UCB-derived cells can be stimulated in an antigen specific fashion to undergo oligoclonal expansion, differentiation into various memory compartment cell types, as well as produce IFN-γ and show cytolytic activity\textsuperscript{48}. UCB-derived cells specific for Wilms’ tumor 1, another TAA, are also able to be generated indicating applicability to multiple tumor types\textsuperscript{49}. 
Chapter 2

Materials and Methods

Umbilical cord blood processing

Umbilical cord blood (UCB) was donated by maternity patients of Sunnybrook Hospital, following informed consent as part of an approved Research Ethics Board protocol. UCB was processed on Ficoll-Hypaque (Fisher Scientific) and purified to yield CD34+ and CD34- fractions using magnetic-assisted cell sorting (MACS) (Miltenyi Biotec). A small sample of the UCB was flow cytometrically analyzed to identify HLA-A*0201 allele status, and the remaining MACS purified cells were cryopreserved in freezing media (Synth-a-Freeze Cryopreservation Medium, ThermoFisher Scientific) and stored at -80°C.

CD34+ stem cell preparation

CD34+ fractions were thawed and flow cytometrically stained with anti-CD34 and anti-CD38 antibodies following blocking of Fc receptors using human FcR blocking reagent (Miltenyi Biotec). Cells were passed through a 0.45 μM filter and resuspended in DAPI-FACS buffer: Hanks Buffered Salt Solution (HBSS) (VWR) supplemented with 1% Bovine Serum Albumin (BSA), 0.1% Gentamicin (Life Technologies), 1% Penicillin/Streptomycin (Fisher Scientific) and 2 μM 4',6-diamidino-2-phenylindole (DAPI). The CD34+ CD38- population was flow cytometrically sorted using a BD FACSARIA III cell sorter and collected into Co-Culture Media:
α-Modified Eagle Medium (α-MEM) (Life Technologies) supplemented with 4% human serum (Gemini Bio-Products), 5 ng/mL human IL-7 (Miltenyi Biotec), 5 ng/mL human Stem Cell Factor (Miltenyi Biotec), 5 ng/mL human Flt3 (Miltenyi Biotec).

CD8⁺ T cell harvesting

Untouched CD8⁺ T cells were isolated from thawed single donor CD34⁺ blood fractions using a CD8⁺ MACS kit according to manufacturer’s protocol (Miltenyi Biotec). After isolation, CD8⁺ T cells were stimulated with aAPCs as described below.

aAPCs

Two aAPC lines were graciously provided by Drs. Carl June (University of Pennsylvania) and Naoto Hirano (University Health Network, Toronto). Both lines are K562-derived and express different molecules which support either polyclonal or antigen-specific T cell activation. The first line, “ABC” aAPCs, expresses HLA-A*0201, CD32 (low-affinity human IgG-Fc binding receptor), CD80 and CD83. The second line, “K80” aAPCs, expresses CD32, 4-1BB, CD80 and CD86. Each of the two lines was transduced to express DLL4 yielding a total of four lines: ABC, ABC-DLL4, K80 and K80-DLL4. A summary of the molecules expressed by the different aAPCs is found in Table 1. aAPCs were cultured in 1640 RPMI with L-glutamine (VWR) supplemented with 15% Fetal Bovine Serum (FBS) (Gibco), 1% Penicillin/Streptomycin and 0.1% Gentamicin.
Table 1:

<table>
<thead>
<tr>
<th>aAPC Line Name</th>
<th>Molecules Expressed/ Secreted</th>
<th>Type of Stimulation Supported</th>
</tr>
</thead>
<tbody>
<tr>
<td>K80</td>
<td>CD80, CD86, CD137 Ligand*, CD32</td>
<td>Polyclonal in presence of anti-CD3ε</td>
</tr>
<tr>
<td>K80-DLL4</td>
<td>CD80, CD86, CD137 Ligand, CD32, DLL4</td>
<td></td>
</tr>
<tr>
<td>ABC</td>
<td>CD80, CD83, HLA-A*0201, CD32</td>
<td>Antigen specific in presence of pulsed peptide</td>
</tr>
<tr>
<td>ABC-DLL4</td>
<td>CD80, CD83, HLA-A*0201, CD32, DLL4</td>
<td></td>
</tr>
</tbody>
</table>

*also known as 4-1BB Ligand.
aAPC transduction

Two day supernatant from the AM12 packaging line with retrovirus containing the DLL4 construct (detectable by GFP expression) was harvested and used to resuspend ABC aAPCs. K80 and K80-DLL4 aAPCs were generated previously in the lab and thus were already available before the beginning of the project. The cells were plated in 24-well flat bottom polystyrene plates at 10⁵/well, centrifuged and incubated at 37°C. Supernatant was removed and replaced with fresh retrovirus-containing supernatant in the afternoon following initial suspension and twice daily thereafter for three days. Three days following the final transduction, GFP⁺ cells were flow cytometrically sorted for high and intermediate cell surface expression of DLL4.

T cell development from CD34⁺ CD38⁻ stem cells

ABC, ABC-DLL4, K80 and K80-DLL4 aAPCs were irradiated (11 291.02 Gy), resuspended in co-culture media and seeded in sterile round bottom 96-well plates. Following seeding of aAPCs, CD34⁺ CD38⁻ hematopoietic stem/progenitor cells (HSPCs) were added to the wells. Cultures were harvested and analyzed by flow cytometry roughly every 7-10 days, at which time fresh irradiated aAPCs were added. As the cells expanded, they were reseeded in progressively larger wells according to cell number.
CD8$^+$ T cell activation

MACS purified untouched CD8$^+$ T cells were counted and suspended in RPMI 1640 with L-glutamine supplemented with 10% Human Serum at $10^6$/mL. K80 and K80-DLL4 aAPC were irradiated and incubated with anti-CD3ε (OKT3, eBioscience) at indicated concentrations for approximately 5 minutes at room temperature. After incubation, aAPC were combined with CD8$^+$ T cells at a ratio of 1:2 and flow cytometric staining was conducted on days 1, 2 and 3 after stimulation.

For activation in the presence of anti-CD27 antibody, aAPCs were incubated with functional purified anti-CD27 antibody (LG.7F9, eBioscience) for 5 minutes at room temperature following a 5-minute incubation with OKT3. aAPCs were then combined with CD8$^+$ T cells and analyzed as above.

Flow cytometry

Antibodies against the following antigens were purchased and utilized according to manufacturer’s suggested concentrations: CD1a, CD5, CD7, CD25, CD34, CD38, CD45, CD71, DLL4. List of clones and manufacturers can be found in Table 2. All samples were incubated with FcR blocking reagent for 15 minutes at 4°C prior to staining for 30 minutes with appropriate antibodies and suspended in DAPI-FACS after staining. Samples were analyzed using an LSRII cytometer (BD Biosciences) using FACS DiVA software and resultant data was analyzing using FlowJo (Treestar).
Table 2: Flow cytometry antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>APC</td>
<td>HI149</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD5</td>
<td>FITC</td>
<td>UCHT2</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD7</td>
<td>PE</td>
<td>M-T701</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD8</td>
<td>AF-700</td>
<td>OKT8</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7</td>
<td>RPA-T8</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD25</td>
<td>BB515</td>
<td>2A3</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>APC-Cy7</td>
<td>M-A251</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>AF-700</td>
<td>M-A251</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD34</td>
<td>PE-Cy7</td>
<td>4H11</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>FITC</td>
<td>581</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD38</td>
<td>APC</td>
<td>HIT2</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD45</td>
<td>APC-eFluor 780</td>
<td>HI30</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD71</td>
<td>APC</td>
<td>M-A712</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>M-A712</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>DLL4</td>
<td>APC</td>
<td>YW152F</td>
<td>Genentech</td>
</tr>
</tbody>
</table>
Quantitative RT-PCR

Total RNA was extracted using Trizol according to manufacturer’s instructions (Life Technologies) from CD8+ T cells that had been cultured with aAPCs at the indicated concentrations and positively enriched using MACS on day 1 after stimulation. Following RNA purification, first strand synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions. Samples were loaded in triplicate and amplified using Power SYBR Green PCR Master Mix (ThermoFisher Scientific) and detected using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers were obtained from Sigma-Aldrich to determine expression of GAPDH, Hes-1, Deltex-1, T-bet and Granzyme A and the sequences can be found in Table 3.

Statistical analyses

Unpaired student’s t-test analysis was conducted to determine statistical significance of results with $p < 0.05$. 

Table 3: q RT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCTGTTCGACAGTCAGCCG</td>
<td>CGACCAATCCGTGACTCC</td>
</tr>
<tr>
<td>Hes-1</td>
<td>AGGCGGACATTCTGGAAATG</td>
<td>CGGTACTTCCCCAGCACACTT</td>
</tr>
<tr>
<td>Deltex-1</td>
<td>CATCCGATCGTCTATGACATC</td>
<td>GATAGCAGTGCGAGGGAATC</td>
</tr>
<tr>
<td>T-bet</td>
<td>CAAGGGGGCGTCCAAACAT</td>
<td>TCTGGCTCTCCGCGTTTCA</td>
</tr>
<tr>
<td>Granzyme A</td>
<td>TCCTATAGATTTTCTGGCATCCTCTC</td>
<td>TTCCTCAATAATTTTTTCACAGACA</td>
</tr>
</tbody>
</table>
Chapter 3

Results

aAPCs can be efficiently transduced to express biologically functional human DLL4.

Retrovirally transduced aAPCs were flow cytometrically sorted to yield cells expressing intermediate and high cell surface levels of DLL4, termed DLL4\textsuperscript{int} and DLL4\textsuperscript{high}, respectively (Figure 4a). Both populations were expanded and cryopreserved, however, for all experiments herein only the DLL4\textsuperscript{high} aAPCs were used, referred to as simply DLL4\textsuperscript{+}. Cell surface expression of DLL4 was maintained reliably despite regular passaging (Figures 4b). Nonetheless, in order to avoid accumulating changes in cell lines over time, fresh aAPCs were thawed every 3-4 weeks. Expression alone of a Notch ligand does not imply this function and so in order to test whether the DLL4 on the aAPCs is able to induce effective Notch receptor signaling, we combined UCB-derived CD34\textsuperscript{+} CD38\textsuperscript{-} HSPCs, with DLL4\textsuperscript{-} and DLL4\textsuperscript{+} aAPCs and used flow cytometry to assess for the induction of T-lineage development (Figure 4c). The current standard protocol for in vitro generation of T cells from HSPCs utilizes OP9 cells, which are a mouse stromal cell line that is able to support development of human T-lineage from HSPCs when OP9 cells are transduced to express DLL4. In the absence of DLL4, developing progenitors are unable to commit to the T-lineage and instead give rise to alternative lineages, such as B or myeloid cells. OP9 cells are adherent while aAPCs are freely suspended in culture, which is an important consideration in that close proximity between the HSPCs
and ligand-bearing cells is required in order to provide the sustained Notch signaling that is required for developmental programming and may be a caveat in this experimental approach. To my knowledge, this is the first study attempting to differentiate HSPCs into T cells using aAPCs as the ligand-expressing cells, and so in absence of an established protocol the aAPC to stem cell ratio was empirically determined so that it would provide the most exposure to DLL4.

The earliest T cell developmental stages are marked by acquisition of CD7 expression, followed by CD1a and CD5 and so these markers were examined to determine differentiation progression. Flow cytometric analysis indicates similar expression levels of DLL4 on the ABC DLL4 and K80-DLL4 cell lines and thus we expected both lines to support differentiation with similar efficiencies (Figure 4d). Within a week, CD34+ CD7+ cells emerge in the co-cultures containing DLL4+ aAPCs, indicating acquisition of the T-lineage by these cells, which now display a progenitor T cell phenotype (Figure 4e). Notably, K80-DLL4 aAPC appeared to be superior in supporting the development of CD7+ cells as this culture featured a more prominent frequency of CD7-expressing cells. As expected, CD34 expression decreased over time, indicating a loss of progenitor cells and this was more prominent in the K80-DLL4 culture. While the expression of CD7 and CD34 changed in ways that are expected in differentiating HSPCs, the expression of CD1a and CD5 did not reflect conventional differentiation. CD7 was consistently expressed in an increasing fashion from days 8 through 25, however, there was no similar large scale acquisition of CD1a and CD5 on the cell surfaces. CD1a appeared to be expressed at low levels in K80-DLL4 cultures by Day 12 and a CD5+ population emerged also in the K80-DLL4 culture at Day 25.
of CD7−CD1a+ cells, which we hypothesize to be of myeloid origin indicating that the HSPCs were able to adopt alternative lineage fates. As expected, in the absence of DLL4 on aAPCs, the developing progenitor cells did not acquire CD7 expression to levels seen in DLL4-containing cultures. Our results suggest that transduced DLL4 is functional and that this system is able to support early stages of T cell development and does not readily provide the necessary molecules for progression to the further stages.
Figure 4: Generation and validation of aAPC lines. (a) Transduced ABC aAPCs were flow cytometrically sorted to yield DLL4<sup>intermediate</sup> and DLL4<sup>high</sup> populations, cells were pregated on GFP<sup>+</sup> before sorting based on DLL4 expression. (b) aAPC lines express DLL4, HLA-A*0201 and GFP despite repeated passaging. (c) Protocol for differentiation of CD34<sup>+</sup> CD38<sup>-</sup> stem cells using aAPCs. (d) Levels of GFP and DLL4 are the same in ABC DLL4 (red) and K80DLL4 (blue) aAPCs. (e) Regulation of expression of CD34, CD7, CD1a and CD5 of developing T cells (pregated on CD45<sup>+</sup>).
DLL4 expression by aAPCs leads to increased frequencies of CD25^+ and CD71^+ CD8^+ T cells during early priming and activation

As the use of aAPCs has predominantly been explored in the expansion of lymphocytes in tumor immunotherapy strategies, we set out to determine whether DLL4 on aAPCs could enhance the suitability of resultant lymphocytes for immunotherapy given reports on the importance of Notch signaling in mature peripheral T cell activation and function. Previously, others have shown that DLL4 is required for activation and anti-tumor function of mouse CD4^+ T cells and that this effect was seen best in limiting antigen concentrations. To determine whether this is also the case in human CD8^+ T cells, we polyclonally activated UCB-derived CD8^+ T cells with a range of OKT3 concentrations presented on K80 or K80-DLL4 aAPCs and assessed for the expression of CD25 and CD71 to determine T cell activation and evidence of Notch signaling, respectively (Figure 5a). In other CD4^+ T cell studies, increasing concentrations of anti-CD3ε antibody led to increased expression of NICD and this effect was sensitive to GSI treatment. With this in mind, we sought to investigate whether this would occur in a human CD8^+ T cell setting. As noted with mouse CD4^+ T cells, we observed effects of DLL4 best at the lowest concentrations of OKT3 one day following stimulation (Figures 5b and 5c). At 0.1 and 5 ng/mL of OKT3, the frequencies of CD25^+ and CD71^+ T cells were significantly higher in the presence of DLL4 on aAPCs, while at higher concentrations no such effect was observed. By days 2 and 3 post stimulation, this effect seemed to dissipate at 5 ng/mL and appears to be sustained at 0.1 ng/mL. However, there was variability in our experimental findings as other experiments showed less conservation of the effect at days 2
and 3 as seen in a graphical summary of two experiments (Figure 5d). The pattern suggests that Notch signaling is affecting CD8+ T cells at a point very early in the activation program and is in line with published observations that show a role for Notch 1 and 2 in early T cell activation processes such as the transition from early effector cell to SLEC22.
Figure 5: DLL4 aAPCs affect early activation program of CD8 T cells. (a) Protocol for CD8 T cell stimulation using aAPCs. (b) Higher frequencies of CD25 and CD71 CD8 T cells are observed on Day 1 post-stimulation in low OKT3 concentration settings but seem to not be present on Days 2 and 3 (pre-gated on CD8α⁺). (c) Summary graphical representation of flow cytometry data in (b).
A titration of OKT3 used to activate T cells showed that the effect was most prominent at and around 5 ng/mL, which was confirmed by further experiments exploring this concentration one day post stimulation. Figures 6a and 6b depict the frequencies of CD25+ and CD71+ cells in two donor bloods stimulated with K80 and K80-DLL4 aAPCs at 5 ng/mL OKT3 which confirms effects seen in an OKT3 titration study. Using CD71 as a proxy, we observed similar conservation of the effect of DLL4 which was most clear on Day 1 post stimulation and dissipated by day 3 at 4.8 ng/mL while remaining at lower OKT3 concentration conditions (Figures 6c and 6d).
Figure 6: Stimulation of more donor bloods with aAPCs at 5 ng/mL OKT3 confirms effect of DLL4 in low activation setting. (a) Higher frequencies of CD25^+ and CD71^+ CD8 T cells in DLL4^+ cultures are observed in cytometry profiles of two different blood donors. (b) Summary graphs of data in (a). (c) Titration of OKT3 investigating concentrations near 5 ng/mL recapitulates trend seen previously. (d) Summary graphs of data in (c).
Variable effect of DLL4 on CD8$^+$ T cell activation in different UCB samples

During the course of completing multiple replicates to assess the effects of DLL4 expression on aAPCs, I noted that the effect of DLL4 seen in the 0.1~10 ng/mL range and was a “moving phenomenon”. For every donor blood that yielded results in line with our observed effects of DLL4, there were others that did not show consistency of these effects. An example of this is depicted in Figures 7a and 7b. The difference in frequency of CD25$^+$ and CD71$^+$ cells at ~5 ng/mL appears to be roughly two-fold in Figure 6b and significantly less so in Figure 7b, which suggests there may be a critical donor blood-specific factor involved in this phenomenon. Further exploration is needed to determine any factors that can be causing these differences.
Figure 7: Effect of DLL4 is not seen in consistent fashion in every stimulated donor. Flow cytometry (a) and graphical summary data (b) of results not in line with those previously shown.
Stimulation of CD27 leads to further increased frequencies of CD25$^+$ and CD71$^+$ cells in presence of DLL4 on aAPCs.

Given that the effect of DLL4 depends on CD28 co-stimulation in mouse CD4$^+$ T cells$^{26}$, we aimed to determine whether the effect of DLL4 observed could be also dependent and/or enhanced by an analogous co-stimulatory pathway. Given that the effect of DLL4 depends on CD28 co-stimulation in mouse CD4$^+$ T cells$^{26}$, we aimed to determine whether the effect of DLL4 observed could be also dependent and/or enhanced by an analogous co-stimulatory pathway and considered other potential costimulatory molecules, such as CD27. To this end, CD8$^+$ T cells were activated in the presence of 5 ng/mL OKT3 and increasing concentrations of functional anti-CD27 agonistic antibody to determine whether the CD27/CD70 pathway could be the relevant co-stimulatory pathway required for an effect of DLL4 in human CD8$^+$ T cells. One day post-stimulation, in the absence of OKT3, there were no increases in frequencies of CD25$^+$ or CD71$^+$ cells regardless of whether the CD8$^+$ T cells were stimulated using K80 or K80-DLL4 aAPCs (Figure 8a). However, in the presence of 5 ng/mL OKT3, the frequencies of CD25$^+$ and CD71$^+$ increased with an increasing concentration of anti-CD27. The magnitude of this increase was notably larger in cultures containing K80-DLL4 aAPCs. There was also evidence of previous experimental inconsistencies in these experiments. While in Donor 1, the frequencies of CD25$^+$ and CD71$^+$ cells are higher at 5 ng/mL OKT3 and 0 ng/mL anti-CD27 as in Figures 5 and 6, this difference is not seen in Donor 2 (Figure 8b). Furthermore, a comparison of the response of two donors illuminates a distinct difference in the range of frequencies of CD25$^+$ and CD71$^+$ cells (~11-22% for Donor 1 and ~2-7% for Donor
2). Additionally, these donors appear to behave differently in response to anti-CD27 antibody with Donor 1 reaching a peak in CD25+ and CD71+ cell frequency at 1 ng/mL anti-CD27 while Donor 2 shows no positive effect at 1 ng/mL, rather reaches a peak at 100 ng/mL anti-CD27. However, CD8+ T cells from either donor revealed that the additional signals from CD27 further augmented the increased in CD25+ and CD71+ cell frequencies seen in the presence of DLL4 expression by the aAPCs.
Figure 8: Functional anti-CD27 antibody enhances effect of DLL4. (a) CD8⁺ T cells were stimulated with 0 or 5 ng/mL OKT3 in the presence of increasing concentration of anti-CD27 antibody and frequencies of CD25⁺ and CD71⁺ cells were analyzed one day post-stimulation, pre-gated on CD8⁺. (b) Graphical summary of flow cytometric analysis corresponding to two blood donors, flow cytometry data in (a) corresponds to “Donor 2” in (b).
Effect of DLL4 in T cell transcriptional changes during priming and activation

Finally, I aimed to characterize early transcriptional changes induced by DLL4 during activation. In order to better understand the effects of DLL4 on CD8+ T cell activation, I conducted qPCR analysis of genes relevant to Notch signaling, cytotoxicity and memory. This panel of genes was designed to detect upregulation of classical Notch targets such as Hes-1 and Deltex-1 and effector genes such as T-bet and Granzyme A. The HES (hairy and enhancer of split) family of basic helix-loop-helix proteins and Deltex family of E3 ligases are classical Notch target genes required for T-cell development and signaling. Furthermore T-bet is also a Notch target gene and activated mouse CD8+ T cells express T-bet which is decreased in the presence of GSI. Overall, I observed no significant up-regulation of Hes-1 and Deltex-1 at 14.4 ng/mL OKT3 or at the 0 ng/mL control. While lower concentrations of OKT3 (4.8 and 9.6 ng/mL) were also investigated in these experiments, they did not yield consistent and detectable expression results (Figure 9). Given that expression of the genes that as I was able to detect were very low as well, it is possible that low starting RNA amounts may be responsible for these difficulties.
Figure 9: qPCR analysis of gene expression induction in cultures stimulated with K80 or K80DLL4 at 0 and 14.4 ng/mL OKT3 one day post stimulation.
Chapter 4

Discussion

The goal of this study was to investigate whether Notch signaling in the context of aAPC-mediated stimulation plays a role in enhancing human CD8\(^+\) T cell activation and function. My findings add DLL4 to the list of molecules that can be stably and functionally expressed by aAPCs, which can be utilized for both inducing T cell development and optimizing co-stimulation to T cells activated for immunotherapy applications. The consistent acquisition of CD7 by HSPCs cultured on DLL4\(^+\) aAPCs is evidence that transduced DLL4 expressed on aAPCs is able to induce Notch signals. Furthermore, our findings suggest that K80-DLL4 aAPCs are better at supporting T cell development than ABC-DLL4 aAPCs, and further investigation of other differences between these two cell lines may shed light on previously unknown requirements for T cell development. Although this experiment was designed to confirm functionality of DLL4, it may also contribute to the wider field of T cell developmental biology in that we have found a system that also supports T cell development. DLL4-expressing OP9 cells are considered the gold standard for T cell development due not only to the functionality of DLL4 on the cell surface, but also to a variety of factors produced by the cells both secreted and on the cell surface\(^{50}\). This repertoire of molecules has not yet been extensively characterized, however, knowledge of other cells able to support T cell development in a similar but distinct fashion is useful in comparatively deducing which molecules are critical for supporting T cell development and at which stages considering early T cell development occurs in an aAPC-based system. An important use of this system, however, could be to create
a clinically relevant and safe therapeutic approach to treating patients with immunodeficiencies. T cell products generated through OP9 cell co-cultures are unlikely to be administered to patients due to the mouse origin of the OP9 cells and potential for accidental injection into humans in instances of impure lymphocyte purification. Manipulation of K562 cells, which are human, to express other molecules critical for T cell development and/or human analogues of molecules produced by OP9 cells is a therapeutic avenue with potential for success that would be safer for human therapeutic purposes as seen in human immunotherapy studies utilizing K562 aAPCs\textsuperscript{37,51}.

The major conclusion of our experiments is that naïve human cord blood CD8\(^+\) T cells are sensitive to Notch signaling during priming and activation and this effect is sensitive to concurrent CD27 signaling. This is evidenced by the observation that the frequency of cells expressing CD25 and CD71 in cultures containing DLL4\(^+\) aAPCs is higher than in those containing DLL4\(^-\) aAPCs and can be increased in the presence of CD27 with a stronger increase occurring in the presence of DLL4\(^+\) aAPCs. Of note, this effect is best seen in low TCR/CD3 stimulation settings, as has been observed in mouse CD4\(^+\) T cells stimulated by APCs expressing DLL4\textsuperscript{26}. In light of the dependency of the effect of DLL4 on CD28 co-stimulation in mouse CD4\(^+\) T cells, our findings suggest that the effect of DLL4 in human CD8\(^+\) T cells is enhanced by CD27 signaling. An early study on CD27 co-stimulation in T cell activation using functional antibody reported a four-fold increase in proliferation in the presence of anti-CD27 agonistic antibody in suboptimal activation conditions and our findings in a limiting activation setting are in line with this study\textsuperscript{52}. Additionally, the effect we observe occurs most prominently within 24 hours of stimulation, while at higher concentrations of TCR/CD3
stimulation this effect dissipates after this period of time. Plausible mechanisms of this phenomenon include a potential additive effect of DLL4 on the CD8+ T cell priming program whereby the transcription of CD25 and CD71 is up-regulated due to Notch signaling in addition to that induced by TCR ligation. Alternatively, it is possible that Notch is lowering the threshold of TCR/CD3 activation, as shown in mouse CD4+ T cells, thus resulting in a higher frequency of activated cells. CD71 is a Notch target gene and the frequency of CD71+ cells was increased in cultures containing DLL4+ aAPCs. This suggests that the DLL4 on aAPCs is functional and able to ligate Notch in a conventional way as evidenced by the expression of a Notch target. Although there is a sizable frequency of CD71+ aAPCs in cultures containing DLL4- aAPCs, this can potentially be attributed to the Notch signaling that is also activated downstream of TCR stimulation. An alternative explanation for higher frequencies of CD71+ T cells in DLL4 aAPC cultures is that DLL4 may be able to cause up-regulation of CD71 independently of TCR activation-mediated Notch signaling. CD28 has also been found to increase sensitivity to low antigen concentrations and so Notch is likely another molecule capable of this effect. This is congruent with our findings since a TCR-modulatory or co-stimulatory role of Notch would be best seen when the TCR signal is low as observed in low OKT3 concentrations. Furthermore, others have shown that GSI treatment causes decreased expression of CD25 and IFN-γ, most notably occurring after 48 hours of stimulation which is consistent with our observations of CD25 expression. We observed a consistent effect on CD25 and given the findings of many other groups, I hypothesize that it is due to the presence of DLL4 on the aAPCs. Others have shown that T cells from Notch1 conditionally-deleted mice have lower numbers of CD25-expressing T cells and lower levels of IFN-γ, and those T cells in
which Notch 1 failed to be deleted expressed high levels of CD25\textsuperscript{21}. It has also been found that GSI moderately reduces CD25 expression dose-dependently\textsuperscript{11}. Conversely, another study reported that in mouse CD8\textsuperscript{+} T cells, GSI treatment had no effect on CD25 expression\textsuperscript{22, 29}. Evidently, the proposed role of CD25 in the effects of Notch on CD8 T cells requires further investigation. Kinetic studies on CD25 expression could be done in this system to determine the downregulation of CD25 over time as it has been shown that CD8\textsuperscript{+} T cells that sustain CD25 expression for longer periods of time preferentially differentiated into terminally-differentiated effectors\textsuperscript{55}.

Our negative qPCR findings are not in line with our flow cytometric data and further experiments should be performed in order to elucidate the cause of this inconsistency. For example, repetition of the experimental set-up using established effector cells, which are also sensitive to Notch signaling but are likely more transcriptionally active may clarify the transcriptional effect of Notch. This is further emphasized by the fact that the literature strongly supports the notion that Notch is involved in transcription of the genes I examined. For example, it has been found that RBPJ binds directly to the Tbx21 (T-bet) promoter, while others have found evidence of non-canonical Notch transcriptional regulation of T-bet via NFkB\textsuperscript{8, 56}. Eomes and T-bet are hypothesized to function together in a coordinated fashion in IFN-γ production and cytolytic functions\textsuperscript{57}. Importantly, memory cells in TBX21-deficient mice are not only enriched but function better than wild type control memory cells in a bacterial infection model and Notch-mediated modulation of this axis would be highly desirable for sustained immunotherapies\textsuperscript{58}. Absence of T-bet results in more central-memory CD8\textsuperscript{+} T cells and fewer effector-memory CD8\textsuperscript{+} T cells\textsuperscript{58}. This is supported by observations that enhanced
T-bet expression is observed in cells that appear to be differentiating into terminally differentiated cells\textsuperscript{58}. Additionally, the Perforin and Granzyme B promoters have GSI-sensitive Notch binding sites, indicating that Notch is an intrinsic regulator of effector CD8\textsuperscript{+} T cell function\textsuperscript{29}. Interestingly, Notch was not shown to govern the expression of FasL indicating that Notch is only partly responsible for the effector CD8\textsuperscript{+} program\textsuperscript{29}. Alternatively, it is possible that the effects we observed are due to DLL4-mediated activation of a pathway existing in the naïve CD8 T cell steady state. This would explain the observation of an effect of DLL4 prior to induction of transcriptional changes.

Association of Notch with non-RBPJ\textsubscript{k} proteins, such as NFkB, in the nucleus is termed “non-canonical”\textsuperscript{21} Notch signaling. It has been proposed by others that conflicting results in Notch signaling effects on T cell activation could be due to RBPJ\textsubscript{k}-independent functions of Notch, which suggests that future studies reporting Notch effects should incorporate mechanistic analysis\textsuperscript{21}. There is also ample evidence that Notch and the NFkB pathways converge to some degree. NFkB signaling is induced downstream of TCR and co-stimulation activation, and among other effects results in upregulation of IFN-\(\gamma\) and CD25\textsuperscript{59}. Furthermore, NFkB signaling is sustained by Notch after 24-48 hours in splenocytes stimulated with anti-CD3\textsubscript{ε} and anti-CD28 antibodies\textsuperscript{59}. Additionally, CD27 induces NFkB signaling\textsuperscript{30} and our experiments show a preferential increase in activated cell frequencies in the presence of DLL4 on aAPCs and responsive to concentrations of anti-CD27 antibody. Finally, the NICD of Notch1 directly interacts with p50 and c-Rel NFkB subunits in 293T cells resulting in expression of NFkB target genes, lengthening of nuclear retention of these subunits and regulation of IFN-\(\gamma\).
production\textsuperscript{59}. Thus the contribution of TCR and Notch signaling to CD25 and CD71 expression should be clarified.

Given the ample evidence in the literature supporting a role for Notch in peripheral T cell transcriptional changes, we hypothesize that our experimental set-up was not conducive to observing these effects. Furthermore, the low cell numbers utilized to set up these experiments (~100 000 CD8\(^+\) T cells per condition), may be contributing to the difficulty in detecting these genes due to low available RNA, especially if initial transcription of genes of interest is low. It is possible that one day is not enough time to observe significant upregulation of these genes particularly in UCB-derived CD8\(^+\) T cells which are hyporesponsive compared to adult blood cells\textsuperscript{60}. CD4\(^+\) T cells derived from UCB show decreased activation markers such as CD69 and CD40 ligand upon activation, as well as reduced production of IFN-\(\gamma\) both with PMA-Ionomycin and CD3/CD28 antibody stimulation and allogeneic DC stimulation\textsuperscript{61}. This is hypothesized to be due in part to reduced AP1 induction and increased expression of anergy genes compared to adult blood-derived CD4 T cells\textsuperscript{61}. Thus it is possible that UCB cells are not reactive enough to see transcriptional effects of Notch signaling on naïve T cell priming and activation. This may also explain inconsistencies among different donor blood results as the degree of hyporesponsiveness may vary from blood to blood. The difference in responses to two donors to anti-CD27 antibody lends further support to this hypothesis. While the effect of DLL4 is increased in both donors in the presence of anti-CD27, the concentrations required to reach the peak increase differ by two orders of magnitude. I currently hypothesize that the lymphocytes in Donor 2 are more hyporesponsive compared to Donor 1 and are a likely explanation for the difference. The
frequency of activated lymphocytes in Donor 1 is considerably higher than in Donor 2 suggesting that Donor 1 is more easily activated and thus having activated “signal 1” in entirety would be more responsive to “signal 2”. The concentration of OKT3 used in the experiment would have been quite unfavourable for activation of Donor 2 lymphocytes, which would necessitate a much higher concentration of anti-CD27 to elicit a similar increase in CD25+ and CD71+ cell frequencies. Given that T cells begin to express CD70 themselves upon activation30, an alternative explanation could be that the more responsive blood experienced up-regulation of CD70 which induced CD27 signaling in a T cell/T cell interaction. Examination of the expression of CD70 on the T cells in future replicates of this experiment would rule out this alternative and confirm that the observed effects were due to the anti-CD27 antibody as opposed to endogenously expressed CD70. In mouse T cells activated in vitro with anti-CD3 or lipopolysaccharide and anti-CD40, the expression of CD70 was “barely detectable” on day 2 post-stimulation and when mice were infected with influenza, the frequency of CD70+ lung-infiltrating T cells was less than 0.5% on Day 3 post-infection62 thus substantial T cell-derived CD70 is likely not available for CD27 ligation within 24 hours of stimulation. I have also tried to expand UCB-derived CD8+ T cells in an antigen-specific fashion, but was unable to do so. The hyporesponsive state of T cells from UCB may reconcile the differences I observed between our results and those published in the literature which, for the most part, use adult human or mouse blood cells. Also, the frequency of fetal- versus adult-derived immune cells is known to vary significantly in different UCB samples63. Perhaps, it may be more informative to perform the experiments using TILs or adult-derived naïve
cells. Also, most if not all studies on Notch in CD8+ T cells use either mouse, adult or transgenic T cells which are not representative of a truly naïve state of cord blood cells.

Future experiments will need to be conducted in order to definitively characterize the effect of Notch on naïve CD8 T cell priming and activation. It is possible that the magnitude of the effect is proportional to the degree of antigen experience or necessitates a particular activating cell type or environment. For example, naïve human Mart-1 specific T cells derived from adult blood and activated using DCs showed reduced outgrowth in the presence of GSI or DLL4-Fc18. This effect could have been facilitated by either the activation history of the cells, or a specific activation environment provided by the DCs. We have tried similar experiments, which did not yield similar results; perhaps the expression of DLL4 on aAPCs does not accurately mirror that in nature either spatially or temporally. Alternatively, differences may be due to differential expression of Notch receptors as it has been shown that naïve T cells express only Notch 2 but effector cells express Notch 1 and Notch218.

Finally, it is possible that I did not utilize the correct Notch ligand to activate the correct Notch receptor that can affect UCB CD8+ T cells. The literature points to DLL4 and DLL1 as the main targets in CD4+ and CD8+ T cells and variation in the studies suggests that the cell type or activation conditions are important. For example, in mouse CD4+ T cells, DLL4 induced the strongest Notch signaling, followed by DLL1 and Jagged164. While DLL4 caused an increased frequency of CD69+ cells, DLL1 and Jagged1 caused a decreased frequency of CD69+ cells64. DLL4 also supported increased average number of cell divisions compared to DLL1 and Jagged164.
Evidently, the role of Notch in peripheral immune cell function is not yet clear-cut and may be subject to modulation by additional pathways in the cell. Figure 10 is a summary schematic of the described findings reported here and in the literature in mouse and human studies examining the role of Notch in T cell activation and function. Better-defined activation systems and starting lymphocyte populations may shed light on the differences that are imparting differential experimental outcomes.
Figure 9: Notch signaling in T cell activation

Figure 10: Antigen presenting cells (APCs) increase expression of Notch ligands, CD80 and CD83 upon toll-like receptor (TLR) activation. CD70 expression is induced upon TLR activation and CD40 signaling. Upon engaging a class I human leukocyte antigen (HLA) molecule presenting a peptide (pHLA-I), T cell receptor (TCR) signaling activates mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and Calcium (Ca$^{2+}$) signaling pathways and Notch signaling. Together with CD28 signaling, TCR signaling activates phosphoinositide 3-kinase (PI3K) which is also activated by CD25 (IL-2 receptor). Both Notch and CD25 signaling preferentially promote differentiation of SLEC versus MPEC. Notch, TCR and IL-2 signaling induce PD-1 expression. CD27 signals via tumor necrosis factor receptor-associated factor 2 and 5 (TRAF2 and TRAF 5) and results in activation of NFκB, interleukin-2 (IL-2) production. In the nucleus, Notch induces “canonical” signaling through association with CSL and “non-canonical” signaling through association with NFκB. NFκB signaling is also promoted by Notch signaling via retention of NFκB in the nucleus. Notch signaling induces the transcription of Hes-1, Deltex1, CD25, T-bet, Granzyme B (GZB), Notch itself, Interferon-γ (Ifng) and PD-1 (Pd1).
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>aAPC</td>
<td>Artificial Antigen Presenting Cell</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive Cell Transfer</td>
</tr>
<tr>
<td>AB</td>
<td>Adult Blood</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankyrin Domain</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-Lymphocyte Associated Protein 4</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CR</td>
<td>Cysteine-Rich</td>
</tr>
<tr>
<td>DLL1</td>
<td>Delta-like 1</td>
</tr>
<tr>
<td>DLL4</td>
<td>Delta-like 4</td>
</tr>
<tr>
<td>DN</td>
<td>Double Negative</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta/Serrate/Lag</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>Hes-1</td>
<td>Hairy and Enhancer of Split-1</td>
</tr>
<tr>
<td>HD</td>
<td>Heterodimerization Domain</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>LFA-3</td>
<td>Lymphocyte Function-Associated Antigen 3</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>MAML1</td>
<td>Mastermind-like1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Melan-A or Mart-1</td>
<td>Melanoma antigen recognized by T cells</td>
</tr>
<tr>
<td>MPEC</td>
<td>Memory Precursor Effector Cell</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch Intracellular Domain</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Sequence</td>
</tr>
<tr>
<td>NRR</td>
<td>Negative Regulatory Region</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PEST Domain</td>
<td>Proline-, Glutamate-, Serine-, and Threonine-rich Domain</td>
</tr>
<tr>
<td>RAM</td>
<td>RBP-J-associated Molecule Domain</td>
</tr>
<tr>
<td>REP</td>
<td>Rapid Expansion Protocol</td>
</tr>
<tr>
<td>SLEC</td>
<td>Short Lived Effector Cell</td>
</tr>
<tr>
<td>SP</td>
<td>Single Positive</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-Associated Antigen</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation Domain</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour Infiltrating Lymphocyte</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TSP</td>
<td>Thymic Seeding Progenitor</td>
</tr>
<tr>
<td>UCB</td>
<td>Umbilical Cord Blood</td>
</tr>
</tbody>
</table>
References


